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Research Article
Effect of Increasing Levels of Dietary Hemp Seed Cake on Egg Quality in Commercial Laying Hens

[1]Rajasekhar Kasula, [1]Fausto Solis, [2]Byron Shaffer, [2]Frank Connett, [2]Chris Barrett, [2]Rodney Cocker and [3]Eric Willinghan

[1]Wenger Animal Nutrient and Technology Innovation Center, The Wenger Group, 101 West Harrisburg Ave, Rheems, PA 17570, USA
[2]Kreider Farms, 1461 Lancaster Rd, Manheim, PA 17545, USA
[3]10119 Berlin-Mitte, Germany

Abstract
Background and Objective: Hemp seed and hemp seed products such as Hemp Seed Cake (HSC) have shown to increase unsaturated fatty acid (FA) profile in eggs, including linoleic acid, known to increase egg weight and α-linolenic fatty acids. However, the use of hemp products in animal feed is still a concern due to the potential residues of the of Δ-9 tetrahydrocannabinol, a psychoactive substance present in the hemp plant. No significant published research is available on the effect of dietary HSC on egg quality parameters in commercial laying hens. The objectives of this study was to determine the effect of dietary HSC on egg quality, external (egg weight, egg mass, eggshell strength, eggshell thickness) and internal (Haugh units, egg yolk pigmentation, egg lutein, egg fatty acids, egg heavy metals and egg cannabinoid residues). **Materials and Methods:** Eight hundred (800) Bovan caged hens in lay at 30 weeks of age were distributed into 4 treatments of 200 hens per treatment based on inclusion levels (0, 10, 20 and 30%) of hemp seed cake (HSC). Each treatment comprised of 8 cages of 25 hens each that served as replicates. The observations per protocol were made over a period of 16 weeks following a 3-week acclimation. **Results:** HSC feeding to commercial laying hens did not adversely affect egg weigh, egg mass; however, positive effects of HSC supplementation was observed on eggshell strength and the polyunsaturated fatty acids including linoleic and linolenic fatty acids. HSC also improved egg lutein, yolk pigmentation and Haugh units. The cannabinoids residues in eggs was below the detectable level. **Conclusion:** The results of this study confirm that HSC fed to laying hens enhanced the overall value of the eggs with increased deposition of beneficial unsaturated fatty acids, yolk pigmentation, Haugh units and lutein content and the trial also demonstrated that feeding HSC to laying hens did not contribute to tetrahydrocannabinol (THC) or cannabinoid residues in eggs.

Key words: Hemp seed cake, eggs, tetrahydrocannabinol, cannabinoids, laying hens

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Corresponding Author: Fausto Solis, Wenger Animal Nutrient and Technology Innovation Center, 101 West Harrisburg Ave, P.O. Box 26, Rheems, PA 17570 , USA Tel: 717-361-4211, 717-917-7545, 1-800-692-6008

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hemp (*Cannabis sativa* L.) is an annual herbaceous plant belonging to the family *Cannabinaceae*[1] , traditionally grown for fiber and seed production. Whole hemp seed contains approximately 25% crude protein, 33-35% oil and 34% carbohydrate, in addition to a broad range of vitamins and minerals[2-4]. Hemp seed oil contains 75-80% polyunsaturated fatty acids (PUFA), including 60% linoleic acid and 17-19% α-linolenic acid (ALA)[5]. The nutrient composition of hemp products provides evidence that these products may serve as potentially valuable livestock feed ingredients.

In the past, the cultivation of hemp was prohibited due to the high content of Δ-9 tetrahydrocannabinol (THC), a psychoactive substance present in the hemp plant. In the recent decades, regulatory changes undertaken by several countries across the globe allowed for the legal cultivation of industrial hemp under a license that permits plants and plant parts of the genera Cannabis, the leaves and flowering heads of which do not contain more than 0.3% THC (wt/wt) and includes the derivatives of such plants and plant parts.

The use of hemp seed cake (HSC) has not been approved in diets for any class of livestock in the USA due to a lack of research in support of its safety and efficacy. There is not much published research available on the effect of feeding HSC on the egg quality.

Objectives: The current study was designed with an objective of determining the effect of increasing levels (10, 20 and 30%) of dietary HSC on external egg quality parameters such as -egg weight, egg mass, eggshell strength and eggshell thickness; and internal egg quality parameters such as - Haugh unit, egg yolk pigmentation, egg lutein content, egg fatty acid profile, egg heavy metal profile and cannabinoid residues.

MATERIALS AND METHODS

Experimental design: The study was conducted at a commercial layer farm in Lancaster County, PA. A part of the commercial layer farm was ear marked for the study. Eight hundred (800) Bovan white caged hens in lay, 30 weeks of age, were distributed into 4 treatments of 200 hens per treatment based on inclusion levels of HSC, as follows: Control diet (C0)- regular diet with no HSC, (H10) - regular diet with 10% HSC, (H20) - regular diet with 20% HSC, (H30) - regular diet with 30% HSC. Each treatment was comprised of 8 cages of 25 hens each that served as replicates. The observations per protocol were made over a period of 16 weeks following a 3-week acclimation.

Acclimation of test animals: In order to eliminate the impact of the new ingredient and its differential inclusion levels, the hens under study were subjected to a period of acclimation for 3 weeks when the respective treatments were fed with the study diets allowing for acclimation of feed consumption and gut environment. Observations and data from the period of acclimation were not considered for the purpose of this study.

Environment and management: All the hens under study were subjected to the uniform environmental and management as follows. Special feed troughs were designed to bypass the existing auto-feeders and the hens were fed manually once a day. An iso-caloric, iso-nitrogenous diet at 25lb/100 hens per day consumption as per breed standard was designed across all treatments. Continuous water, identical environment and management were offered uniformly across treatments. Hens were weighed prior to start of study by cage and composition of hens per cage was managed for uniformity of body weight across treatments. Environmental conditions were maintained at 74-76°F house temperature, 40-60% humidity, 30 Lux lighting for 15-16 h of lighting per day and air movement between 2550 and 3400 m^3 h^{-1} per 1000 hens.

In order to establish uniformity of population across treatments, the cages were individually weighed for initial weights and, hens moved between cages so as to maintain a total body weight difference not exceeding 2.5%. These weight-adjusted cages were then randomized within the 32 cage locations with 2 cages of same treatment together. A plastic plate was installed between each cage thus preventing hens from picking feed from adjacent cage feeder.

Nutritional composition of HSC and finished feed: The analysis of the nutritional composition of HSC and the study feeds formulated with HSC are presented in Table 1 and the formulation of the feed is presented in Table 2.

Heavy metals in HSC and experimental diets: The levels of heavy metals arsenic, cadmium and lead in HSC and experimental diets are reported in Table 3. The levels of heavy metals in HSC were below laboratory detectable levels. The control ration showed significantly higher levels of arsenic and cadmium over HSC diets. The lead profiles of experimental rations did not vary significantly.

Feeding program: Study hens were offered a uniform restricted amount of feed at 25lb/100 hens per day across all treatments. A pre-weighed 6.25lb of feed was provided to each cage of 25 hens every day at the same time. At this level,

Table 1: Hemp seed cake and Feed nutritional analysis (%)

HSC/treatments	HSC	SD	C0	SD	H10	SD	H20	SD	H30	SD
Moisture	7.53	0.31	12.12	0.01	11.21	0.38	10.03	0.47	8.40	0.20
Protein (crude)	32.06	0.30	14.81	0.51	16.31	0.19	16.75	0.06	16.57	0.25
Fat (crude)	9.02	0.03	2.70	0.00	5.57	0.05	8.78	0.26	11.47	0.16
Fiber (crude)	32.21	0.44	1.79	0.11	4.92	0.87	7.07	0.18	9.82	0.11
Ash	5.38	0.05	11.27	0.21	11.48	0.28	12.71	0.04	12.21	0.55
Minerals (%)										
Ca	0.17	0.01	3.38	0.03	3.18	0.08	3.61	0.24	3.45	0.14
P	0.71	0.47	0.50	0.06	0.50	0.01	0.56	0.04	0.57	0.01
Na	0.01	0.00	0.14	0.01	0.14	0.01	0.16	0.01	0.15	0.01
Mg	0.48	0.01	0.17	0.01	0.21	0.00	0.26	0.01	0.28	0.00
Mn (ppm)	133.00	0.58	78.50	3.54	93.55	1.77	135.00	9.90	145.00	7.07
Fe (ppm)	133.67	2.01	283.50	38.89	260.00	7.07	261.50	13.44	244.00	12.21
Zn (ppm)	77.83	0.56	86.15	7.85	89.60	4.53	123.50	10.61	128.00	2.83
Cu (ppm)	18.83	0.46	19.40	0.28	17.55	0.35	17.95	0.07	19.20	3.54
K	0.95	0.02	0.73	0.05	0.72	0.01	0.73	0.04	0.62	0.00
Amino acids (%)										
Methionine	0.51	0.12	0.42	0.10	0.42	0.01	0.44	0.10	0.52	0.01
Cysteine	0.34	0.05	0.24	0.04	0.23	0.00	0.22	0.02	0.24	0.01
Lysine	1.13	0.02	0.86	0.05	1.04	0.05	1.00	0.05	0.97	0.16
Phenylalanine	1.24	0.01	0.72	0.02	0.81	0.01	0.71	0.00	0.75	0.00
Leucine	1.93	0.02	1.34	0.03	1.45	0.03	1.25	0.01	1.29	0.00
Isoleucine	0.91	0.01	0.52	0.02	0.69	0.02	0.52	0.01	0.61	0.01
Threonine	1.18	0.03	0.59	0.07	0.72	0.01	0.67	0.02	0.66	0.06
Valine	1.13	0.02	0.57	0.03	0.77	0.01	0.61	0.02	0.76	0.01
Histidine	0.73	0.02	0.41	0.02	0.50	0.01	0.41	0.00	0.48	0.00
Arginine	4.00	0.05	0.93	0.06	1.26	0.01	1.39	0.02	1.82	0.04
Aspartic acid	1.37	0.03	1.60	0.13	1.63	0.02	1.76	0.00	1.56	0.11
Serine	3.55	0.03	0.82	0.07	0.87	0.05	0.82	0.02	0.77	0.05
Glutamic acid	1.45	0.02	2.73	0.23	2.70	0.01	2.75	0.03	2.46	0.23
Proline	4.94	0.03	1.07	0.06	1.03	0.02	0.99	0.01	0.98	0.06
Hydroxyproline	1.35	0.04	0.13	0.01	0.08	0.01	0.17	0.01	0.14	0.00
Alanine	1.16	0.01	0.78	0.05	0.84	0.01	0.70	0.04	0.78	0.01
Tyrosine	0.89	0.01	0.51	0.01	0.54	0.01	0.50	0.01	0.51	0.01
Tryptophan	0.27	0.00	0.10	0.01	0.11	0.01	0.19	0.01	0.13	0.01
Fatty acids (%)										
Oleic 18:1 w7	1.05	0.01	0.80	0.00	1.16	0.01	1.21	0.01	1.26	0.01
Linoleic 18:2 w6	55.26	0.05	55.30	0.16	54.59	0.23	54.73	0.10	54.91	0.04
Linolenic 18:3 w6	3.43	0.02	0.00	0.00	0.45	0.01	0.69	0.02	0.81	0.01
Linolenic 18:3 w3	14.47	0.05	2.66	0.15	6.01	0.00	7.33	0.16	8.00	0.11
Total% W3	15.34	0.06	2.66	0.15	6.10	0.00	7.63	0.16	8.23	0.12
Total% W6	58.69	0.06	55.30	0.16	55.03	0.23	55.51	0.12	55.72	0.06

Data are the mean of three replicates (n = 3) of HSC and two replicates (n = 2) of each feed type, SD: Standard deviation

it was expected that the hens consumed nutrients per breed recommendation for the age and stage of production.

Preparation of composite egg sample: A specific composite sampling procedure was followed for analyzing certain parameters of egg quality, that included of the following steps:

- Collect 3 eggs from each of the 8 cages of the treatment under process, a total of 24 eggs per treatment
- Prepare 3 sets of 8 eggs each with 1 egg representing each of the cages
- Break the 8 eggs from each set, mix and homogenize the whole egg contents for a minute with an egg homogenizer (easy mix mixer-bowl rest feature of 5 speed), pour in a sterile plastic bottle previously identified with details of treatment. This makes 1 composite sample
- Prepare 3 such composite samples per treatment
- Repeat the procedure for other treatments

Study parameters, test and analytical methods

External egg quality parameters

Egg weight (g per egg): Egg weight was determined as a mean of 8 replicates (n = 8) per treatment. Ten eggs per replicate (8 replicates per treatment×10 eggs per replicate = 80 eggs per treatment) were weighed. Egg weight was performed once a week. No grading of eggs for size was performed.

Table 2: Study diets formulated by treatment (lb)

Ingredient/treatment	C0	H10	H20	H30
Corn	1304.70	1187.90	1066.70	919.10
Soybean meal- solvent	463.00	334.00	206.00	102.00
Calcium chip	98.00	97.00	98.00	98.00
Limestone	98.00	97.00	98.00	98.00
Monocalcium phosphate 21%	20.40	18.10	15.80	13.30
Salt	5.09	5.13	5.17	5.22
Methionine, DL	4.00	4.00	4.00	3.80
Sodium sesquicarbonate	3.60	3.60	3.60	3.60
Vitamin premix	1.00	1.00	1.00	1.00
Trace minerals premix	1.00	1.00	1.00	1.00
Choline, Liq. 70%	0.62	1.43	2.25	2.97
Alpha-gal 280 P	0.33	0.33	0.33	0.33
Phytase	0.16	0.16	0.16	0.16
HSC	0.00	200.00	400.00	600.00
Soybean oil	0.00	44.00	90.00	139.00
Lysine sulfate 60%	0.00	3.48	6.95	9.28
Tryptophan	0.00	0.49	0.97	1.33
Threonine	0.00	0.40	0.90	1.00
Ingredient total	2000.00	2000.00	2000.00	2000.00
Calculated nutritional composition				
Moisture	11.57	13.32	16.13	17.06
Crude protein	15.86	15.88	15.90	16.34
Fat (Ether extract)	2.65	5.39	8.20	11.16
Crude fiber	1.99	5.01	8.01	11.04
Ash	12.34	11.80	11.79	10.79
Minerals (%)				
Avail Ca	4.17	4.11	4.13	4.12
Avail P	0.44	0.44	0.44	0.44
Na	0.17	0.17	0.17	0.17
Cl	0.195	0.195	0.195	0.195
Poultry ME	1290.23	1290.64	1290.62	1290.39
Amino acids				
Lysine, digestible	0.75	0.76	0.78	0.79
Methionine, dig	0.43	0.43	0.43	0.42
Met & Cys, dig	0.65	0.65	0.64	0.63
Tryptophan, dig	0.17	0.17	0.17	0.16
Threonine, dig	0.53	0.53	0.52	0.52
Glycine, dig	0.59	0.58	0.56	0.57
Phenylalanine, dig	0.74	0.69	0.64	0.61
Leucine, dig	1.32	1.22	1.12	1.05
Histidine, dig	0.40	0.37	0.35	0.34

Table 3: Levels of heavy metals in HSC and experimental diets (mg kg^{-1})

	Feed						
Heavy metals/treatment	HSC	C0	H10	H20	H30	p-value	SD
Arsenic	<0.05	0.20[a]	0.10[b]	0.10[b]	0.10[b]	0.0001	0
Cadmium	<0.05	0.09[a]	0.06[b]	0.06[b]	0.06[b]	0.0001	0
Lead	<0.05	0.20	0.20	0.15	0.20	0.4789	0.04

Data are the mean of three 3 replicates (n = 3) of HSC and 2 samples (n = 2) of feed diets. Means with different superscripts are significantly different (p<0.05)

Egg mass (g hen^{-1} day^{-1}): The egg mass was determined from 8 replicates per treatment (n = 8) as follows:

Egg mass (g of eggs per hen per day) = (mean egg production (%) × livability/100) × egg weight (g)

The egg production was determined for each individual replicate or cage and 8 replicates were analyzed per treatment for statistical analysis. The egg production was adjusted for livability to account for the dead hens. The egg weight was determined per procedure described earlier.

Eggshell strength (g): Eggshell breaking strength was determined at least 24 h after collection at room temperature by quasi-static compression using an Egg Force Reader machine[6]. The eggs were placed horizontally between 2 flat

parallel steel plates and compressed at a speed of 5 cm min^{-1}. Eggshell breaking strength represents the minimum force required to fracture the egg and it was expressed in grams[7]. The eggshell breaking strength was performed on 40 eggs (n = 40) per treatment at the rate of 5 eggs per cage and was recorded on a weekly basis for the entire study.

Eggshell thickness (mm): The eggshell thickness of each egg was recorded as the mean of 3 points of measurement, each at the broad end, equator and narrow end using a digital display micrometer gauge. The eggshell thickness was measured from 16 eggs per treatment (n = 16) collected at the rate of 2 eggs per cage on Day 1 and at the end of week 8 and week 16 of the study.

Internal egg quality parameters:

Haugh unit: The Haugh unit was determined as a mean of 40 eggs (n = 40) per treatment collected at the rate of 5 eggs per cage on Day 1, at the end of week 8 and week 16. Albumen height was measured using a tripod micrometer Ames-56428, (B. C. Ames Co Waltham Mass, USA) with the eggs weighed and cracked on a flat glass balanced surface for the highest point of the albumen closest to the yolk. The Haugh unit was calculated with the following formula:

$$=100\times\log(B-(5.674504384\times(30\times\text{POWER}(C8,0.37)-100)/100)+1.9)$$

Where:

B : Hight of the albumen in mm

C : Weight of the egg in grams

Egg yolk pigmentation: Yolk pigmentation was measured visually with a Vepinsa (also known as Roche) Color Fan as previously reported[8] by matching the color of yolk with the color spectrum of the fan. The yolk pigmentation was determined as a mean of 40 eggs per treatment collected at the rate of 5 eggs per cage on Day 1, at the end of week 8 and week 16.

Egg lutein content (mcg g^{-1}): Using a composite egg sample, as described earlier, lutein was extracted from approximately 1 g of egg sample and frozen at -80°C before HPLC analysis. Three replicate egg composite samples from each treatment were used for statistical analysis and their mean values, standard deviation and statistical significance were reported. The HPLC analysis (Waters 2796, Waters, Milford, MA) for lutein content was performed using a C18 reverse-phase column (3.5 µm, 4.6 mm i.d. ×150 mm length; ×Bridge, Milford, MA). The isocratic mobile phase (100% methanol) was maintained at a flow rate of 1.0 mL min^{-1} and automated injections of 50 µL were made. Absorbance at 445 was monitored using a photodiode detector (Waters 2998, Waters). Millennium software (Waters) was used to process and integrate peaks[9] .

Egg fatty acid profile: Using three composite samples from each treatment the mean fatty acid values were expressed as weight percentages[10,11] along with linoleic to linolenic acid ratio. The fatty acid composition was determined using standard gas chromatographic techniques of the fatty acid methyl esters[12] , using C17:1 fatty acid (Nu-Chek Prep, Inc., Elysian, MN) as an internal standard. Total lipids were extracted from the test diets, egg yolks, breasts and abdominal fat by homogenization in chloroform/methanol (2:1, v/v) according to the methods of Folch *et al.*[13]. After centrifugation, the organic phase was collected and evaporated under a N2 stream. The all lipid extracts obtained were transesterified with methanolysis [1% (v/v) H2SO4 in methanol] for 3 h at 70°C. After cooling, the resulting fatty acid methyl esters (FAMEs) were extracted with hexane and transferred into gas chromatography (GC) vials. All solvents contained 0.005% (v/v) butylated hydroxyanisole (BHA) as an antioxidant. FAMEs were then separated and quantified with a Varian450-GC with CP-8400 autosampler, equipped with a flame ionization detector and a GC column (length 30 m, inner diameter 0.25 mm and film thickness 0.25 µm, DB-225MS) (Agilent Technologies, Mississauga, ON, Canada). Nitrogen was the carrier gas at a column flow rate of 1 mL min^{-1}. The inlet split ratio was set at 10:1. The oven temperature programming was as follows: 60°C for 1.5 min, raised to 180°C at 20°C min^{-1}, 205°C at 6°C min^{-1}, 220°C at 2°C min^{-1} for 4 min and 240°C at 10°C min^{-1} for 3 min. The injector and detector temperature were set at 260 and 290°C, respectively. FAMEs were identified by comparison of retention times to known lipid standards (Nu-Chek Prep, Inc., Elysian, MN)[11,13].

Egg heavy metals: Three composite egg samples from each treatment diet to determine heavy metals by inductively coupled plasma emission spectroscopy (PerkinElmer Optima 2100DV, Wellesley, MA) and quantities were determined based on the reference standards[14].

Egg cannabinoid residues: Using whole egg composite sampling as described earlier, 3 replicate samples per treatment were submitted for the analysis of the residues of various hemp cannabinoids at weeks 8 and 16. The analysis were carried out at Eurofins Laboratory, Madison, WI, method 2018.11, AOAC International (Modified by the procedures by

Lukas *et al.*[15] "Quantification of Cannabinoids in Cannabis Dried Plant Materials, Concentrates and Oils Liquid Chromatography-Diode Array Detection Technique with Optional Mass Spectrometric Detection," First Action Method, Journal of AOAC International, Future Issue, Eurofins *et al.*, 2017, Eurofins Laboratory, Madison, WI, USA).

Statistical analysis: All parameters except the heavy metals for HSC and cannabinoids were analyzed using SAS[16] with a completely randomized design with cage as the experimental unit with the help of the general linear model procedure (PROC GLM). The treatment mean separation was carried out with the Tukey Multiple Range test with a probability of error of 5% ($p<0.05$). Heavy metals of HSC and cannabinoids did not need statistical analysis since no specific levels were recorded as all values were the same, below the laboratory detectable levels.

RESULTS

External egg quality parameters

Egg weight (g): The egg weights from various treatments stayed within acceptable range of breed variance for most part of the study with occasional but inconsistent tendency to increase with inclusion levels of HSC. Certain inconsistency was observed at different time periods, such as, at week 3, there was a reduction from 58.45 at 10% to 56.75 at 30% inclusion of HSC, while at week 12, there was an increase in egg weight from 57.55 in the control treatment to 59.42 g at 10% inclusion of HSC; again at week 16, when the egg weight was reduced from 59.76 in the 10% HSC to 57.83 in the 30% HSC (Table 4).

Egg mass (g hen^{-1} day^{-1}): The egg mass in general showed a numerically downward trend across all treatments, including the control, during the 16 weeks of study, atypical to the breed. There was only one significant difference between the treatments at 1 week. Towards the end of the study, the differences between mean egg mass were statistically non-significant (Table 5).

Eggshell strength (g): The eggshell breaking strength in various treatments are presented in Table 6. With minor inconsistencies, the eggshell breaking strengths of treatments followed the expected declining trend of the breed as the hens aged post-peak. Eggs from control hens had consistently poor breaking strength with those of H30 at week 7, 8, 11 and 12 and with those of H10 at week 11. At the end of the study, the overall mean eggshell strengths showed a tendency to increase, with the 30% being significant at 5107.30 g compared to 4836.20 g in the control (Table 6).

Eggshell thickness (mm): The eggshell thickness was not significantly affected by the supplementation of HSC; the mean eggshell thickness stayed at 0.37 in all treatments, including the control and those fed with HSC (Table 7).

Internal egg quality parameters

Haugh unit: The key internal egg quality represented by the Haugh units showed a positive impact of feeding HSC. The observations followed the typical reduction trend of breed post-peak production. However, at week 8 the Haugh units in all HSC treatments were significantly higher compared to the control with a similar non-significant trend at week 16. The

Table 4: Effect of feeding increasing levels of HSC on egg weight (g)

Treatment/week	C0	H10	H20	H30	p-value	SD
1	56.24^{ab}	57.32^{a}	56.13^{ab}	55.61^{b}	0.03	1.11
2	57.43	57.21	57.20	56.13	0.13	1.15
3	58.00^{ab}	58.45^{a}	57.26^{ab}	56.75^{b}	0.01	1.04
4	56.98	57.32	57.21	56.35	0.58	1.49
5	57.26	57.38	56.41	56.35	0.27	1.32
6	57.26	56.81	56.75	57.37	0.25	0.74
7	57.09	57.49	56.69	57.09	0.65	1.24
8	57.83	57.49	57.54	57.32	0.91	1.39
9	56.92	57.77	57.77	57.37	0.43	1.17
10	56.92	58.45	57.83	57.26	0.18	1.44
11	57.43	57.60	57.09	57.66	0.84	1.37
12	57.55^{b}	59.42^{a}	57.66^{ab}	58.34^{ab}	0.04	1.36
13	57.94	59.02	58.68	58.34	0.52	1.48
14	58.28	58.96	57.60	58.34	0.13	1.10
15	58.45	59.10	57.09	58.11	0.14	1.74
16	58.90^{ab}	59.76^{a}	58.74^{ab}	57.83^{b}	0.03	1.20
Mean	57.53	58.10	57.35	57.26	0.06	0.65

Data are the mean of eight replicates (n = 8) per treatment

Table 5: Effect of feeding increasing levels of HSC on egg mass (g hen^{-1} day^{-1})

Treatment/week	C0	H10	H20	H30	p-value	SD
1	50.38^{b}	53.60^{a}	52.58^{ab}	51.01^{a}	0.02	2.18
2	53.00	53.50	52.99	51.52	0.37	2.32
3	53.31	54.43	53.43	52.22	0.18	1.95
4	50.70	52.14	51.86	50.48	0.55	2.75
5	51.13	53.45	52.27	51.37	0.22	2.38
6	50.83	51.31	51.59	52.17	0.52	1.79
7	49.27	50.50	50.28	49.75	0.85	3.01
8	49.24	49.04	48.76	49.98	0.80	2.56
9	45.91	48.04	46.10	49.44	0.09	3.05
10	44.39	45.09	43.68	48.46	0.22	4.80
11	42.31	43.87	41.58	46.66	0.24	5.19
12	40.84	45.31	40.01	44.71	0.14	5 .35
13	40.28	43.64	39.70	45.66	0.17	5.94
14	40.70	42.77	38.41	44.62	0.13	5.30
15	40.70	41.50	36.85	44.45	0.07	5.52
16	38.49	38.93	37.16	40.85	0.29	3.76
Mean	46.34	47.94	46.08	48.33	0.23	2.59

Data are the mean of eight replicates (n = 8) per treatment, Means with different superscripts are significantly different (p<0.05)

Table 6: Effect of feeding increasing levels of HSC on eggshell strength (g)

Treatment/week	C0	H10	H20	H30	p-value	SD
1	5265.13	5164.63	5009.28	5175.33	0.523	774.89
2	5021.00	4934.73	5103.10	5179.03	0.455	711.35
3	4892.50	5066.30	5149.60	5279.60	0.158	773.46
4	4810.75	4825.68	5073.63	5075.98	0.076	613.21
5	4977.18	4981.43	5045.43	5252.03	0.268	709.69
6	4954.03	4978.40	4952.70	5039.70	0.948	744.90
7	4799.25^{b}	5033.48^{ab}	4933.35^{ab}	5331.43^{a}	0.011	726.72
8	4834.03^{b}	5147.15^{ab}	5231.75^{ab}	5358.83^{a}	0.013	732.28
9	4711.63	4998.03	4937.18	5091.43	0.200	816.90
10	5035.98	4859.53	4783.93	4909.48	0.592	838.67
11	4637.8^{b}	5051.65^{a}	4896.4^{ab}	5089.43^{a}	0.035	755.47
12	4723.78^{a}	4957.38^{ab}	4749.98^{a}	5156.05^{b}	0.051	784.77
13	4691.88^{b}	4705.95^{ab}	4773.5^{ab}	5139.58^{a}	0.016	706.30
14	4861.53^{a}	4939.03^{a}	5076.65^{a}	4857.88^{a}	0.642	864.46
15	4647.68^{a}	4727.35^{ab}	4981.68^{b}	4999.48^{b}	0.062	712.63
16	4514.35^{a}	4810.85^{a}	4585.95^{a}	4779.93^{a}	0.212	745.09
Mean	4836.20^{b}	4948.95^{b}	4955.30^{b}	5107.30^{a}	0.0001	218.84

Data are the mean of 5 eggs per cage×8 cages = 40 eggs) (n = 40) per treatment. Means with different superscripts are significantly different (p<0.05)

Table 7: Effect of feeding increasing levels of HSC on eggshell thickness (mm)

Treatment/week	1	8	16	Mean
C0	0.37	0.37	0.37	0.37
H10	0.37	0.37	0.37	0.37
H20	0.37	0.38	0.37	0.37
H30	0.38	0.37	0.37	0.37
P-Value	0.80	0.66	0.97	0.81
SD	0.02	0.02	0.023	0.02

Data are the mean of 2 eggs per cage (2×8 cages: 16 eggs) (n = 16) per treatment. Means with different superscripts are significantly different (p<0.05)

overall mean Haugh units of both time periods showed a significant increase at 10 and 20% HSC inclusion compared to control group (Table 8).

Egg yolk pigmentation: The yolk pigmentation scores of eggs showed a positive impact of HSC feeding although the trend was inconsistent. Towards the end of study, the overall mean yolk pigmentation scores were significantly higher in all HSC fed hens over control group at 6.93. H10 at 7.56, H20 at 7.43 and H30 at 7.46, although the differences between HSC fed hens stayed non-significant (Table 9). The scale of egg pigmentation ranges from a minimum of 1 (less pigmented) to a maximum of 15 (highest pigmentation) (DSM, formerly Roche fan).

Egg lutein content: The mean lutein content of egg samples measured at the end of the study is presented in Table 10. The observations showed a positive and statistically significant correlation between lutein content and HSC inclusion levels.

Table 8: Effect of feeding increasing levels of HSC on Haugh units

Treatment/week	8	16	Mean
C0	90.4500^{b}	86.95	88.860^{b}
H10	93.7800^{a}	88.94	91.440^{a}
H20	94.1300^{a}	89.77	91.950^{a}
H30	92.4800^{a}	88.13	90.430^{ab}
P-Value	0.0009	0.30	0.007
SD	4.3200	6.23	4.130

Data are the mean of 5 eggs per cage (5×8 cages= 40 eggs) (n = 40) per treatment. Means with different superscripts are significantly different (p<0.05)

Table 9: Effect of feeding increasing levels of HSC on egg yolk pigmentation

Treatment/week	8	16	Mean
C0	6.7100^{b}	7.140	6.9300^{b}
H10	7.5400^{a}	7.580	7.5600^{a}
H20	7.3800^{a}	7.470	7.4300^{a}
H30	7.3100^{a}	7.610	7.4600^{a}
P-value	0.0001	0.446	0.0001
SD	0.6000	7.550	3.3600

The scale of egg pigmentation ranges from a minimum of 1 and a maximum of 15. Data are the mean of 5 eggs per cage (5×8 cages = 40 eggs (n = 40) per treatment. Means with different superscripts are significantly different (p<0.05)

Table 10: Effect of feeding increasing levels of HSC on egg lutein content (mcg g^{-1})

Treatment/week	16
C0	2.9400^{d}
H10	4.4800^{c}
H20	5.7800^{b}
H30	6.5600^{a}
P-value	0.0001
SD	0.2240

Data are the mean of three composite replicates (n=3) per treatment. Means with different superscripts are significantly different (p<0.05)

Egg fatty acid profile: The results of mean fatty acid profiles of eggs at the end of 16th week as presented in Table 11 showed significant influence of feeding HSC as follows:

Total fatty acids significantly increased over control at 20% inclusion level of HSC although the differences were numerically higher at 10 and 30% inclusion level.

Omega 3 and 6 fatty acids significantly increased over control with increasing levels of HSC with a similar reduction in Omega 9.

The Polyunsaturated fatty acid (PUFA), Linoleic acid (LA) and alpha-linolec acid (ALA) significantly increased over control with increasing levels of HSC supplementation. A significant corresponding reduction in LA:ALA ratio was noticed with greater inclusion levels.

The levels of monounsaturated fatty acids (MUFA) showed a significantly reducing trend over control with increasing levels of HSC except at 10% inclusion level which showed only a numerical reduction.

The total cis-fatty acid levels in all HSC fed groups were significantly higher over control and showed an increasing trend with HSC inclusion which was statistically significant except between 20% and 30% inclusion level of HSC.

Egg heavy metals: The concentration of heavy metals in eggs determined at the end of study were below laboratory detectable levels in all treatments, including control (Table 12).

Egg cannabinoid residues: The mean cannabinoid residue levels, including delta-9-tetrahydrocannabinol and cannabidiol were below laboratory detectable levels which is 0.0025% for egg samples (Table 13). The cannabinoid and

Table 11: Effect of feeding increasing levels of HSC on egg fatty acids (%) at week 16

Fatty acid/Treatment	C0	H10	H20	H30	p-value	SD
Saturated fatty acids	3.39	3.39	3.48	3.18	0.0670	0.1170
Total cis fatty acids	5.32^{c}	5.68^{b}	6.00^{a}	6.02^{a}	0.0020	0.1530
Mono-unsaturated fatty acids (MUFA)	3.87^{a}	3.39^{b}	3.23^{b}	2.87^{c}	0.0001	0.1230
Polyunsaturated fatty acids (PUFA)	1.45^{d}	2.29^{c}	2.78^{b}	3.15^{a}	0.0001	0.1180
Omega-3	0.070^{d}	0.220^{c}	0.290^{b}	0.346^{a}	0.0001	0.0130
Omega-6	1.45^{d}	2.18^{c}	2.61^{b}	2.94^{a}	0.0001	0.1100
Omega-9	3.53^{a}	3.18^{b}	3.06^{b}	2.77^{c}	0.0003	0.1160
Total fatty acids	9.18^{a}	9.53^{ab}	9.97^{b}	9.65^{ab}	0.0480	0.2790
Linoleic acid (LA)	1.15^{a}	1.87^{b}	2.28^{c}	2.63^{d}	0.0001	0.1020
α-Linolenic acid (ALA)	0.03^{a}	0.10^{b}	0.14^{c}	0.20^{d}	0.0001	0.0094
LA: ALA ratio	45.56^{a}	18.86^{b}	15.99^{c}	13.48^{d}	0.0001	1.7100

Data are the mean of three composite replicates (n = 3) per treatment. Means with different superscripts are significantly different (p<0.05)

Table 12: Effect of feeding increasing levels of HSC on egg heavy metals

Treatments	Units	Arsenic	Cadmium	Lead	Mercury
C0	Ppb	<10	<5	<5	<5
H10	Ppb	<10	<5	<5	<5
H20	Ppb	<10	<5	<5	<5
H30	ppb	<10	<5	<5	<5

Data are the mean of three composite replicates (n = 3) per treatment at every week, SD: 0

Table 13: Effect of feeding increasing levels of HSC on hemp cannabinoid residues in eggs (<%)

	Week 8				Week 16			
Week/treatments	C0	H10	H20	H30	C0	H10	H20	H30
THCA	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
CBC	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
THCVA	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
CBNA	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
CBCA	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
CBL	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
*Total THC	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
**Total CBD	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025

SD: 0. *Total THC (THC+(THCAx0.877), **Total CBD (CBD+(CBDAx0.877). Data are the mean of three composite (n = 3) replicates per treatment. Means with different superscripts are significantly different (p<0.05)

related component levels of eggs in HSC treated hens were not different from those of control tested at both intervals of the study.

DISCUSSION

Most of the published literature on the effect of dietary hemp seed cake is on other species and with using whole hemp seed, hemp oil or other hemp products. Extremely limited published researches are available regarding the effect of feeding HSC on egg quality in commercial laying hens, the authors are constrained with few supporting references to quote on the findings.

Effect on external egg quality: In the current study, although at week 3, the external egg quality parameters appeared to show differences, the treatment difference was not significant over the control group. Inconsistent findings have been reported by researchers with hemp seed meal[17], hemp seed[18] and hemp seed cake[19], who reported that hemp products supported egg weights at certain levels, contrary to Neijat *et al.*[20], whose studies with hemp seed, showed that hens fed 30% had a significantly lower egg weight over control or the lower levels.

Additionally, the egg mass in general showed a reduction atypical of the breed across all groups including the control, during the 16 weeks of study with no trend or pattern that was statistically significant. The authors have not found any published research in support of or contradiction to this finding.

The eggshell strength showed a positive trend that was statistically significant while the shell thickness showed none between the treatments. This finding is in line with Tatara *et al.*[21] who opined that mechanical endurance of the eggshell is not simply affected by its thickness but other factors such as mineral density, mineral content and spatial micro architectural arrangement contribute to this characteristic. The mean eggshell thickness among various groups in the current study falls under medium category, in which researchers Tatara *et al.*[21]., Mohamed and Tůmová[22], reported no positive correlation between eggshell thickness and eggshell strength. The finding about the beneficial effect of HSC on eggshell strength is an addition to the current knowledge pool and could not be cross verified for want of related published literature.

Effect on internal egg quality

Effect on Haugh units: The Haugh Units of HSC fed hens in all treatments stayed higher than that of the control group but did not differ with increasing levels. However, researchers with hemp seed[18] and hemp seed meal[19] reported no significant differences in Haugh units in their 4 week investigations. The longer feeding period showed the benefits of feeding HSC.

Effect on yolk pigmentation: Consistent enhancement of egg yolk pigmentation with increasing levels of HSC has been an impressive finding in the current study. Similar findings were reported by Goldberg *et al.*[23] who used hemp seed and hemp oil. Skřivan *et al.*[24] also used the hemp seed and found the similar results, he also added, that the increase in color intensity of egg yolks did not adversely affect the sensory profiles of cooked eggs. A large segment of consumers prefers deep pigmented eggs not only from an esthetic perspective but also for the benefits of carotenoids to vision[25,26]. Due to these benefits to human health, scientists have paid much attention to xanthophyll and in particular the roles of lutein and zeaxanthin in prevention of certain eye disorders[27].

Effect on lutein: The current study showed a positive response in lutein enrichment of eggs with feeding HSC that was statistically significant. This result is consistent with a previous study conducted by Skřivan *et al.*[24] who researched with hemp seed. Landrum *et al.*[28] reported that the optical density of the macular pigment increased by 30% in humans due to lutein supplementation, which equates to a 40% reduction in the amount of blue light that reaches the retina.

Eggs fatty acid composition: The prime perceived nutritional value of HSC as an alternative animal feed ingredient is its superior fatty acid composition, with a high contribution of unsaturated and omega fatty acids. The general positive trend in total fatty acid levels, a strong Omega 3 and 6 fatty acid levels, polyunsaturated fatty acids (PUFA), linoleic and linolenic acid levels, cis-fatty acids and trends of reduction in saturated fatty acid levels, monounsaturated fatty acid (MUFA) levels and linoleic: alpha-linoleic ratios in egg and abdominal fat, only confirm the beneficial effects of feeding HSC. This result reinforces the findings of a study by Gakhar *et al.*[18] who used hemp seed and Silversides in his study. Similar findings were reported by Silversides and Lefrancois[19] who used hemp seed meal.

The high unsaturated fatty acid and essential fatty acid (Omega 3 and 6) levels in eggs may be attributed to their high levels in HSC. This, reduction in Omega 9 and saturated fatty acids enhance the nutritional profile of eggs. Omega-9 fatty acids (including oleic acid and erucic acid) unlike omega-3 and omega 6 are not considered essential fatty acids.

Egg heavy metals: Additionally, the study also observed that the heavy metals in eggs (arsenic, cadmium, lead and mercury) were below detectable levels. This finding is an addition to the current knowledge pool of HSC feeding safety to laying hens and could not be cross verified for want of related published literature.

Egg cannabinoid residues: The hemp cannabinoid levels in eggs were reported to be below the detectable levels of 0.0025% by chromatographic methods in the laboratory and were under the legal limits of 0.3%. The primary concern with feeding HSC to animals continues to be the transfer of hemp cannabinoid residues, mainly cannabidiol (CBD) and delta-9-tetrahydrocannabinol. Published research stated that a level of Δ-9 tetrahydrocannabinol (THC), (a psychoactive substance in the hemp plant)[29] below 0.3% is safe for animal feeding[11]. The authors could not cross verify this finding since no published research on transfer of cannabinoids to eggs is available.

CONCLUSION

The current study has sufficiently evaluated and captured the effect of HSC on egg quality in commercial laying hens and concluded that Dietary HSC up to 30% in layer feed did not adversely affect the egg weight and egg mass. Dietary HSC up to 30% improved external egg quality expressed by eggshell strength with no effect on eggshell thickness and internal egg quality as demonstrated by improvement in Haugh units, yolk pigmentation and lutein. Dietary HSC up to 30% enhanced the levels of omega 3 and 6 fatty acid levels and reduced their ratio, moreover, it did not influence the heavy metal and cannabinoid residues profile of eggs.

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